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# SOME CONDITIONS AFFECTING THE GROWTH AND ACTIVITIES OF AZOTOBACTER CHROOCOCCUM


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## INTRODUCTION

The problem of soil biology is concerned to a considerable extent with studies of the activities of the oligocarbophilous and of the oligonitrophilous bacteria. Representatives of both the groups appear to be very widely distributed, and the inference is that they are more or less active in all normal arable soils. Of the former group the *Nitrosomonas, Nitrosococcus,* and the *Nitrobacter* of Winogradsky ("the nitrifying bacteria") are the most widely known, while of the latter group the *Azotobacter* and the *Bacillus radicicola* or legume bacteria ("the nitrogen-fixing bacteria") are the most familiar examples. All of these and related forms have been the subjects of extended research, and consequently an immense and growing literature exists on this general subject.

Although the physiological powers and the presence in ordinary soil of these organisms can be readily proven by suitable incubation experiments with soils or impure culture, attempts to isolate and grow these organisms, especially the *Azotobacter* and the nitrifiers, in synthetic media of entirely known composition lead to very great difficulties. Pure cultures are not readily isolated, and when obtained their growth on media of entirely known composition is so slow that inves-

tigations of the mechanism of their activities have not been particularly inviting problems to most workers. This condition or set of conditions has resulted in the production of a large amount of "soil" and crude culture work and a comparatively small amount of true physiological work. Many students of these problems have contended that this is as it should be; that pure cultures in completely synthetic media are so unnatural that results can have but little practical bearing. It seems to us that this type of reasoning is unsound and that it can never be productive of thoroughly reliable either practical or purely scientific work. This point has been discussed at length by Allen and Bonazzi ('15) with especial reference to the study of nitrification. Existing methods of work were criticized, and the difficulties to be encountered in the improvement of methods discussed. Since then, in line with this method of attack, has appeared the work of Allen ('15) and of Davisson and his co-workers ('16, '18, '19) on improvement of methods of nitrogen determinations, and of Bonazzi ('19, '19[a]) on the nitrifying bacteria. The work reported in this paper deals with experiments on *Azotobacter chroococcum,* and they have proved to be as crude and erratic as were those reported earlier on nitrification, yet just as illustrative of the difficulty of the problem and just as suggestive, we hope, of possible methods of attack.

## HISTORICAL

To review in detail all the difficulties that have been reported in studies of *Azotobacter* since the organism was discovered by Beijerinck in 1901 is wholly unnecessary at this time. From numerous and diverse sources it is evident that ordinary synthetic culture media are lacking in something for pure culture work, and that aqueous soil extract or even tap water is superior to distilled water, but that the addition of a small amount of soil to the culture medium is far better.

A step forward was made by Krzemieniewski ('08), who found that humus was the important constituent of the soil for *Azotobacter,* and that the activating substance in the soil

and more particularly in the soil humus was difficultly soluble in water. The work of Krzemieniewski has rightly been the subject of much discussion, and no wholly satisfactory explanation has been brought forward yet to explain the remarkable results obtained on the addition of humus to Beijerinck's mannite culture solution. The explanations that have been suggested are, however, well worthy of note.

Kaserer ('10) conceived the idea of humus supplying certain rare or unusual inorganic constituents to the culture medium which were required in very small amounts by *Azotobacter,* but entirely essential nevertheless to their proper development. After many experiments with media containing iron, aluminium, manganese, and silicon, he considered that he had very nearly duplicated in a synthetic way the remarkable results of Krzemieniewski, which success he attributed to the presence of iron and aluminium silicophosphates which furnished iron and aluminium in soluble form to the bacteria. He believed, however, that he had not yet attained the best possible combination of required mineral nutrients. Later ('11[a]) he elaborated to a considerable extent on this theory, postulating that all bacteria require these rather unusual elements and compounds to a certain extent, but that the requirements in this line of the oligocarbophilous and of the oligonitrophilous forms were especially high. The decoctions and broths on which the ordinary saprophytic and pathogenic bacteria are ordinarily grown contain sufficient of the above compounds to cover the needs of these organisms, hence their presence has remained undetected. He predicts that before the ideal culture medium is attained minute amounts of other substances will have to be introduced, e. g., copper, zinc, arsenic, iron, and titanium. He also believes that the necessity of these unusual elements for green plants cannot be denied with certainty.

Remy and Rösing ('11) made an extended study of the cause of the results obtained from humus by Krzemieniewski. After confirming certain important points in regard to the beneficial action of humus, they proceeded to search for an explanation for such action. Kaserer's solution containing

iron, aluminium, silicates, and phosphates proved valueless in their hands, but a faintly alkaline mannite solution containing iron and aluminium phosphates appeared promising. Following this out through a series of experiments they finally succeeded in preparing a nutrient medium which contained, in addition to the regular constituents of Beijerinck's nutrient, a so-called "ferric hydroxide solution," with cane sugar as a protection against precipitation ("Fallingsschutz") by alkalis. This iron-containing solution was prepared by dissolving in 1 liter of water 1 gm. of $FeCl_3$, 10 gms. cane sugar, and 0.80 gm. iron-free NaOH. Two drops of $H_2SO_4$ were then added. This final solution contained 0.50 gm. $Fe_2O_3$ and 0.26 gm. free NaOH per liter. Numerous distinctly beneficial results are reported from the addition of $Fe_2O_3$ to Beijerinck's nutrient solution in the above form. For instance, when 15 mgs. $Fe_2O_3$ were added to 100 cc. of Beijerinck's solution 7.88 mgs. of nitrogen were fixed by *Azotobacter* per gm. of mannite in 2 weeks, whereas ordinarily only 1 to 2 mgs. were assimilated per gram of mannite.

By increasing the amount of $Fe_2O_3$ Remy and Rösing state that better results are obtained, although they unfortunately omitted the nitrogen determinations for these larger amounts of iron. The other substances, NaCl, cane sugar, and NaOH, present in the so-called ferric hydroxide solutions, were without effect, hence these workers felt that the following conclusions were justified:

(1) A relation exists between the iron content of the nutrient solution, *Azotobacter* development, and nitrogen fixation. In the case of ferric silicate the optimum lies above 10 mgs. $Fe_2O_3$ per 100 cc. of Beijerinck's mannite solution.

(2) The value of the iron varies according to its form. The most favorable is an alkaline solution in which iron hydroxide is dissolved by means of cane sugar. Then follows ferric silicate, while all other iron compounds stand far below. Thomas phosphate acts strongly, which is to be attributed in part to the content in silicic acid and basic lime.

In discussing the results of their work Remy and Rösing point out that the action of the iron cannot be that of a nutri-

ent, since the optimum lies too high. They call attention to the fact that H. Fischer suggested the rôle of humus in the soil to be that of an oxygen carrier, and that Bonnema suggested that $Fe_2O_3$ is the real agent in nitrogen-fixing processes, since in contact with the air it transforms continually small amounts of nitrogen into nitrite, and that this continually formed nitrite is then transformed by the microörganisms into cell protein. On these points Remy and Rösing state that they would soon offer experimental evidence, but to our knowledge it has not appeared.

Kaserer ('12) believes that the results obtained by Remy and Rösing were due to the impurities in the iron compounds used rather than to the iron itself, but Rösing ('12) does not accept this explanation.

Söhngen ('13) studied to some extent the conditions for promoting the growth of *Azotobacter*, the nitrifying bacteria, and bacteria in general. As concerns *Azotobacter* he states that the results of Krzemieniewski, Kaserer, and Remy and Rösing were in general confirmed. In addition he found that colloidal silicic acid when added to Beijerinck's medium produced an even more beneficial effect than the raw humus which he used. With Beijerinck's solution alone he obtained 1.9 mgs. nitrogen fixed per gm. of mannite, whereas when 2 gms. raw humus were added to 100 cc. of culture solution 6.7 mgs. were fixed, and when 500 mgs. colloidal silicic acid in the sol form were added 8.0 mgs. of nitrogen were fixed per gram of mannite. All the results cited were obtained with a pure culture in a 32-day incubation period.

Söhngen also obtained excellent results by inserting a strip of filter paper or of cotton cloth in the culture medium. *Azotobacter* grew almost exclusively in contact with the filter paper or cloth at or just above the junction of the strips with the nutrient solution. This indicates, according to him, that microbial life in the soil takes place chiefly upon the colloids. He believes that the beneficial action of the colloidal silica and of the raw humus is due to the fact that these colloids adsorb nitrogen and oxygen, and in this manner impart more quickly the necessary elements, and better growth results.

In further confirmation of his theory Söhngen measures the oxygen and nitrogen adsorbed by colloidal silica and colloidal ferric oxide. He finally concludes that for luxuriant development of *Azotobacter* in Beijerinck's medium only nitrogen and oxygen are lacking. By the use of the colloids described by him there occurs a direct contact between the bacteria and oxygen and nitrogen, with the result that luxuriant growth of *Azotobacter* takes place in the culture medium.

Still another theory has been proposed by Bottomley ('14) to account for growth stimulation in cultures of *Azotobacter* and nitrifying bacteria. Marked acceleration of growth of these bacteria and of wheat seedlings was obtained by him by the use of extracts of "bacterized" peat, i. e., peat which had been inoculated with certain aërobic bacteria and incubated for a suitable time under favorable conditions as to temperature and moisture. He later ('15) proposed the term "auximones" for these accessory substances, which he believed were analogous to the "vitamines" of animal physiology. Bottomley's associate, Miss Mockeridge ('17), studied in some detail the action of these extracts of bacterized peat, and believed that "auximones" had been responsible for the marked results obtained by Krzemieniewski on the addition of humus to cultures of *Azotobacter*. Bottomley ('17) claims to have isolated a nucleic acid derivative from "bacterized" peat and suggests that it is of importance in the results obtained on accelerated growth.

Very recently Bonazzi ('19) has obtained results on the growth of nitrifying bacteria that are very much to the point on this general problem. Intensive growth as measured by one physiological activity was obtained in case of *Nitrosococcus* (the nitrite-producing bacteria) when the solution was properly stirred mechanically. The nutrient medium was of very simple composition, so that the possibility of "auximones" and "rare elements" would be eliminated. It was also soon observed that this type of treatment was distinctly beneficial for the growth of *Azotobacter*. Aside from mentioning that the shaking possibly produced better aëration or assisted in the removal of by-products, Bonazzi did not enter into any

speculations as to the cause of the benefit derived from the mechanical stirring.

None of the theories which have been mentioned above will fit all the facts in regard to hastening the growth of *Azotobacter* or of the nitrifying bacteria. These theories have, however, been most suggestive and helpful in furnishing a stimulus to the investigation in this difficult field. It seems to us that the only way to proceed with the formulation of a theory is to keep trying, and to test the theories as they are proposed from as many viewpoints as possible. In this way only is it possible, it seems to us, to avoid the performance of an almost endless amount of empirical work.

Now, in viewing the results obtained from diverse sources on the stimulated growth of *Azotobacter* and of the nitrifiers, we find that in all cases growth is very slow in pure cultures in solutions under normal conditions of completely known composition. There appears to be universal agreement on this point. No one doubts, apparently, on the other hand, that the addition of humus to cultures of *Azotobacter* is quite effective, and that vigorous growth of the nitrifiers may be quite easily produced in all normal soils.

In trying to find a common factor to account for all the various improvements in culture solutions it should be borne in mind that Kaserer obtained his beneficial results from a "silicophosphate," and that Bottomley believes his "auximones" to be a nucleic acid derivative, therefore a carrier of phosphorus. We must bear in mind also that Gerlach and Vogel ('03), after studying the mineral requirements of *Azotobacter chroococcum,* concluded that phosphorus and calcium were absolutely indispensable nutrients. After a 67-day incubation period they found the following nitrogen relations per 1,000 cc. of nutrient solution:

| | Flask content (mgs. N) | Gain over control (mgs. N) |
|---|---|---|
| Series I (all inorganic nutrients) ..... | 45.2 | 42.5 |
| Series II (without calcium)..... | 3.1 | 0.4 |
| Series III (without potassium)..... | 21.6 | 18.9 |
| Series IV (without phosphorus)..... | 2.8 | 0.1 |

| | | Flask content (mgs. N) | Gain over control (mgs. N) |
|---|---|---|---|
| Series V | (without potassium and phosphorus) | 2.9 | 0.2 |
| Series VI | (without sodium) | 18.0 | 15.3 |
| Series VII | (without potassium and sodium) | 21.2 | 19.5 |
| Series VIII | (all nutrients, uninoculated) | 2.7 | |

In addition to these essential elements it is quite evident—although absolutely definite data are lacking—that the reaction of the medium is quite important. It is known beyond any question that the medium must not be acid, but the exact concentration of hydrogen as ion has not been properly studied. It has been more or less generally considered that the reaction should be faintly alkaline, and the fact that the addition of solid calcium carbonate to the culture medium, which is then maintained or "buffered" to a reaction of approximately $P_H$ 8.0, has found quite wide favor, is in accord with this idea.

Now it is evident at once that these three requirements, phosphates, calcium, and a faintly alkaline solution, are very difficult to obtain, owing to the formation of the but slightly soluble tricalcium phosphate. For instance, in Ashby's medium, which is prepared from distilled water, mannite (or dextrose), potassium phosphate, sodium chloride, calcium and magnesium sulphates, a trace of ferric chloride and solid calcium carbonate, the phosphates are essentially quantitatively transformed into the almost insoluble tricalcium phosphate. In Beijerinck's medium, which is made from tap water, mannite (or dextrose), and dipotassium phosphate, the calcium (contained in the tap water) is precipitated during the sterilization processes owing to the hydrolysis of the dipotassium phosphate.[1] Thus Ashby's solution is deficient in soluble phosphates, but is well buffered slightly on the alkaline side, while Beijerinck's solution is lacking in soluble calcium salts, its only buffer being a relatively low amount of dipotassium phosphate, which maintains its reaction approximately at neu-

[1] In Winogradsky's medium for nitrifying bacteria the phosphorus is precipitated as ferric, ferrous, and magnesium phosphates.

trality at incubation temperatures.[1] The addition of calcium carbonate, therefore, to Beijerinck's solution introduces two variables: It changes its reaction and it precipitates the phosphates. It is not surprising, therefore, that the tendency has been to omit calcium carbonate in Beijerinck's solution, especially when mannite is used as the energy source.[2] In Ashby's solution more soluble calcium (as sulphate) is added than in Beijerinck's solution, hence more of the phosphate is carried down, with the result that the solution is very poorly buffered and hence unsuited to the growth of *Azotobacter* (especially when dextrose is used), unless calcium carbonate be supplied to each culture vessel. Although direct comparisons are few or wanting entirely, it seems from the literature that Beijerinck's solution is preferable to Ashby's. In the light of the above reasoning it may easily be that this is because it contains more soluble phosphate than does Ashby's and that this, besides being more available as a nutrient, acts as a soluble buffer and as such is more effective than the solid calcium carbonate.

Now it was conceivable to us that the phosphorus nutrition and possibly the maintenance of proper hydrogen ion concentration separately or in conjunction were operative in all the above-cited cases of growth stimulation of *Azotobacter chroococcum*. For instance, in Krzemieniewski's experiments the organic combinations of calcium may have supplied this element in an assimilable form and organic phosphates in the humus may also have been beneficial. The colloids which Söhngen used might have acted as protective colloids, i.e., prevented the complete flocking out of the tricalcium phosphate after it was once formed. The properties of soil grains which he believed was due to adsorption of gases may have been due to their adsorbed phosphate ions, or possibly to the difficultly soluble phosphate compounds precipitated as a thin film as a

[1] The exact $P_H$ value is influenced by the mannite or the dextrose present.

[2] When dextrose is added it, of course, shifts the reaction slightly toward the acid side, because of its properties as a weak acid. (See Mathews, A. P. Physiological Chemistry, p. 32. 1916.)

part of the coatings of the grains.[1] So far as culture solutions are concerned, however, it seems to us that the action of the colloid as a protection against precipitation of tricalcium phosphate is a simpler explanation.

In considering the work of Kaserer and of Remy and Rösing, particularly of the latter, the fact must not be lost sight of that the terminology used by them with respect to solutions is very loose in the light of modern chemistry. The "ferric hydroxide solution" of Remy and Rösing is, of course, a colloidal suspension of hydrated ferric oxide, and as such it might easily possess the property of a protective colloid and prevent the flocking out of phosphates in the culture medium. Kaserer's postulation of a silicophosphate as a chemical compound is unwarranted. It is much more likely that his "iron aluminium silicophosphate" is a colloidal suspension of varying composition and stability carrying with it some tricalcium phosphate in colloidal suspension.

As concerns Bottomley's results, it has already been mentioned that he considers the benefit observed by him to be due to compounds containing phosphorus in the organic form. Regarding the results obtained by Bonazzi with mechanical agitation, it is clear that "auximones" and "rare elements" are eliminated, although "oxygen carriers" and the "adsorption of gases" might really have the same action as that of the shaker. It seemed more probable to us that the precipitate which formed in the medium contained phosphates absolutely essential to the development of the microörganisms, and that the agitation hastened the restoration of the concentration of the solution in the equilibrium which exists between this solid phase and the nutrient solution as the materials are

[1] Söhngen considers the possibility of adsorption phenomena being operative. For instance, he says: 'The equilibrium between the concentration of the dissolved substances in the soil water and the nonorganized colloids is therefore continually disturbed in consequence of the metabolism of the organized colloids. Yet the concentration of substances in the soil water is maintained more nearly constant than in a medium without colloids, for the reason that the nonorganized colloids give up again the compounds obtained from the soil water because of the new state of equilibrium between the concentration in the liquid and the colloid. . . . . There is therefore a continual exchange of assimilable compounds between the organized and the nonorganized colloids, by virtue of which the mass of the nonorganized colloids serves as a storehouse with assimilable compounds, which is daily filled, and out of which the organized colloids regularly feed.'

assimilated, i. e., removed from solution, by the growing forms.

Thus it seems that phosphorus nutrition is associated more or less intimately with the growth and development of the *Azotobacter*. The problem then to us seems to be in part a question of supplying phosphates in the presence of calcium in a slightly alkaline solution. To test our hypothesis the set of experiments described below has been carried out. The theory is naturally quite difficult to prove or disprove, especially in view of the experimental difficulties encountered in work of this kind, and while our results have not led to positive conclusions one way or another it seemed that in view of the uncertainty of carrying work to completion at the present time it was best to report what findings we have, with the hope that they may be of value as suggestions to others as well as to ourselves.

## Experimental

*Culture used.*—The *Azotobacter* used was a subculture from a strain of *Azotobacter chroococcum* isolated by Mr. A. Bonazzi from Wooster, Ohio, soil. It was repeatedly plated during the isolation until it gave a uniform microscopic picture and produced on Ashby's mannite agar plates circular colonies, edges entire, moderately raised and shiny. Its purity was assured during this work by repeated platings on mannite agar similar to Ashby's in composition. The colonies on agar plates appear, in 24 hours, small, round, and translucent. They grow rather slowly and become opaque, grayish white, and up to 4 or 5 days are of almost butyrous consistency, while as the culture ages they gradually become dry and at 10 days present a slightly wrinkled condition, while at 12 to 14 days the growth is dry, distinctly wrinkled, and shows a faint production of black pigment. The growth of *Azotobacter* on agar slant is entirely similar to the above, although, of course, in consequence of its slimy consistency it shows a fairly marked invasion at the base of the slant. Grown on modified Ashby's soil extract (1:2) mannite agar, it soon acquires a marked pigmenting power, which it loses again when grown on purely

synthetic media. In culture solutions it manifests itself by the formation of a translucent zoögloea mass at the junction of the solution surface and the walls of the container. At from 4 to 6 days this largely disappears and a fine white sediment appears at the base of the flask. In no case did we observe even the suggestion of the surface scum or pellicle which is widely described in the literature.

The purity of the culture also was checked by numerous microscopic examinations. The regular method for making microscopic preparations was staining for 5 minutes with dilute 1:10 aqueous methylene blue, clearing in xylol-alcohol, equal parts, and then in xylol. Preparations were also made, using ordinary carbol gentian violet and clearing as above. The cells from very young cultures (12 hours) show little differentiation in structure when stained with ordinary carbol gentian violet or with dilute aqueous methylene blue, and tend to be oval or bacillary in form. Slightly older cultures (e. g., 60 hours) show some differentiation and the appearance of granules is more frequent. Cells from cultures 4 to 6 days old appear as large, thin-walled, decidedly granular cocci or diplococci, while preparations from old cultures (e. g., 15 days) generally show thick-walled cocci surrounded by considerable slime, the cell contents failing to take either of the above stains appreciably (pl. 1, figs. 1–6). The nature of these cytological differentiations has been discussed by Bonazzi ('15).

The culture when obtained was in its twenty-fifth transfer from soil, and transfers and platings made from this time on were designated as $F_1$, $F_2$, $F_3$, etc.

*Culture medium.*—The standard culture medium used in this work was essentially a modified Ashby's medium of the following composition:

| | | |
|---|---|---|
| Mannite | 20 | gms. |
| Monopotassium phosphate | 0.2 | gm. |
| Magnesium sulphate | 0.2 | gm. |
| Sodium chloride | 0.2 | gm. |
| Calcium sulphate | 0.1 | gm. |
| Distilled water | 1000 | gms. |
| 10% ferric chloride solution | 2 | drops |

Agar was prepared by the addition of 1.5 per cent Bacto[1] agar to the above. Soil extract agar was prepared by substitution of 1:2 soil extract in place of distilled water. A pinch of $CaCO_3$ was added to each agar tube or culture vessel. In platings special care was taken to get the $CaCO_3$ well into suspension before pouring, as growths on the plates were better when this precaution was taken.

Although, as stated above, the Beijerinck medium is probably superior to Ashby's, we chose the latter, as it was of known composition, whereas the use of tap water introduced unknown factors. Beijerinck's solution was used therefore only in a few cases for purposes of comparison.

Our experimental work is best considered under three separate heads: (1) the preliminary or orientation work which is concerned chiefly with the checking of important results; (2) the improvement of experimental methods; and (3) the final experiments which are designed to throw light on the reason for improved growth in cultures of *Azotobacter* mechanically agitated.

## ORIENTATION EXPERIMENTS

It seemed to us that it was well worth while to attempt to duplicate certain phases of Remy and Rösing's work. At the outset we experienced some difficulty in preparing a stable colloidal ferric oxide solution according to the directions of Remy and Rösing. This we attributed to the presence of sulphate ion. After a few empirical experiments, a colloidal ferric oxide solution was prepared as follows: One gm. Kahlbaum's pure ("zur Analyse") $FeCl_3$ and 10 gms. of saccharose were dissolved in approximately 700 cc. of distilled water, 0.34 gm. $Na_2CO_3$ added, the solution heated till perfectly clear, cooled, and made to 1 liter. This solution was then strongly reddish in color, and showed no tendency to precipitate when heated to boiling, although a small portion of it did precipitate when added to Ashby's solution and autoclaved. Assuming that the $FeCl_3$. 6 $H_2O$ was pure, the solution contained .295 gm. $Fe_2O_3$ per liter. This iron-sugar solu-

[1] Prepared by Digestive Ferments Co., Detroit, Mich.

tion was carefully checked in regard to its nitrate content. A nitrate determination on a 5-cc. portion by the modified Devarda method did not give an amount of nitrogen detectable with N/50 acid. A blank solution was prepared containing saccharose and NaCl equivalent to the $FeCl_3$ and added to the different cultures in the amounts indicated below.

Two series of cultures were prepared, the one employing 100 cc. of Ashby's solution in 300-cc. Erlenmeyer flasks, the other the same amount in 700-cc. Erlenmeyers. The culture solution was prepared as indicated above, double distilled water being used. The reaction was carefully adjusted to the phenolphthalein neutral point and approximately ½-gm. portions of c. p. $CaCO_3$ added to each flask. One-mg. and $\frac{1}{10}$-mg. portions of colloidal $Fe_2O_3$ were added to certain of the culture solutions. These amounts were supplied by the addition of the proper amounts of the iron-sugar solution or of dilutions prepared from it. Corresponding amounts of the NaCl sugar solutions were added to the controls.

The culture solutions were inoculated with a suspension prepared from a 72-hour slant of $F_4$ on Ashby's soil extract agar. As much of the growth as could be removed with a spiral was transferred to a 10-cc. water blank, well shaken, and one spiral of the suspension used for inoculating each flask of culture medium. The cultures were incubated for two weeks in a warm room, after which they were analyzed for total nitrogen by the Kjeldahl-Gunning method. The contents of the cultures were transferred to 500-cc. Kjeldahl flasks with ammonia-free water, the complete transfer of the culture material being assisted by the addition of the 30 cc. of concentrated sulphuric acid in three 10-cc. charges to the Erlenmeyer flasks and subsequent washings into the Kjeldahls. Ten gms. of anhydrous sodium sulphate and 2 cc. of 10 per cent copper sulphate solution were added and digestion carried out in the regular manner. Boiling was continued for 1½ hours after the solutions became clear. After cooling the melt was treated with 200 cc. of nitrogen-free water, 60 cc. of 50 per cent alkali then added, and distillation performed with an apparatus essentially the same as that used previously

for nitrate determinations (Allen, '15, fig. 1), N/50 acid being used in the receivers. Bumping was prevented by the addition of zinc.

Extreme difficulty was experienced in carrying out the digestions. Foaming was excessive and the cultures reported "lost" in table 1 were those which foamed over. Only a very low flame could be used and the rate of digestion was extremely slow. The large amount of carbonaceous material, which formed from the decomposition of the mannite, was extremely resistant to digestion, and it was only after several days of slow intermittent boiling that the mixtures cleared. After the determinations on these preliminary experiments were completed, some studies on optimum conditions for digestion were taken up. These are discussed separately.

The results of the first of the preliminary experiments are given in table 1. The control cultures received, as stated above, sodium chloride and cane sugar equivalent to the ferric oxide and cane sugar in the remaining flasks.

TABLE I

EFFECT OF COLLOIDAL FERRIC OXIDE IN FIXATION OF NITROGEN BY AZOTOBACTER CHROOCOCCUM IN DEEP AND SHALLOW LAYERS

| No. | Additions to Ashby's culture medium | Treatment | 300-cc. flasks | | 700-cc. flasks | |
|---|---|---|---|---|---|---|
| | | | N found (mgs.) | N fixed (mgs.) | N found (mgs.) | N fixed (mgs.) |
| 1 | Control | Sterile | Lost | ..... | 1.31 | ..... |
| 2 | Control | Inoc. | 3.23 | 1.56* | 2.59 | 1.28 |
| 3 | Control | Inoc. | 2.84 | 1.17 | 1.98 | 0.67 |
| 4 | 0.1 mg. $Fe_2O_3$ | Sterile | 1.67 | ..... | 1.34 | ..... |
| 5 | 0.1 mg. $Fe_2O_3$ | Inoc. | 5.08 | 3.41 | 7.94 | 6.60 |
| 6 | 0.1 mg. $Fe_2O_3$ | Inoc. | 4.52 | 2.85 | Lost | ..... |
| 7 | Control | Sterile | 1.34 | ..... | 1.45 | ..... |
| 8 | Control | Inoc. | 2.33 | 0.99 | 2.60 | 1.15 |
| 9 | Control | Inoc. | 1.80 | 0.46 | 3.99 | 2.54 |
| 10 | 1.0 mg. $Fe_2O_3$ | Sterile | Lost | ..... | 1.22 | ..... |
| 11 | 1.0 mg. $Fe_2O_3$ | Inoc. | 2.72 | 1.38† | Lost | ..... |
| 12 | 1.0 mg. $Fe_2O_3$ | Inoc. | Lost | ..... | 4.73 | 3.51 |

* Computed from No. 4 as blank. † Computed from No. 7 as blank.

While the data are erratic and incomplete, they indicate that the 0.1-mg. portion colloidal ferric oxide exerted a beneficial effect on the growth of the microörganism. The growth

in the deeper layers is unsatisfactory, as would be expected from the work of others.

The effect of colloidal ferric oxide in Beijerinck's[1] solution was also studied. The nutrient solution contained 20 gms. mannite and 0.2 gm. $KH_2PO_4$ per 1000 cc. of tap water. A portion was titrated with phenolphthalein, and the calculated amount of normal NaOH added to make the medium very faintly alkaline to phenolphthalein. For the sake of determining the effect of tap water, a second medium was prepared in exactly the same manner except that redistilled water was used. One-hundred-cc. portions were pipetted into 700-cc. "Nonsol" Erlenmeyers, and then 1.0 mg. of colloidal $Fe_2O_3$ in the form of the solution described above was added to each flask. Since the concentration of salts is less in the Beijerinck solution than it is in the Ashby, and since it lacks $CaCO_3$ besides, it was reasonable to expect that the colloidal $Fe_2O_3$ would remain in suspension better in it than in the Ashby. While no difference could be detected in the unheated solutions, after autoclaving the Beijerinck medium appeared to be more colored than the Ashby, although partial precipitation occurred in both. The data obtained after a two weeks' incubation at 28–32° C. are reported in table II below. For the sake of comparison the data on Ashby's solution containing 1.0 mg. $Fe_2O_3$ per culture of 100 cc. are retabulated.

TABLE II

GROWTH IN PRESENCE OF 1.0 MG. OF COLLOIDAL $FE_2O_3$ IN DIFFERENT NUTRIENT SOLUTIONS

| No. | Medium | Treatment | Water | N found (mgs.) | N fixed (mgs.) |
|---|---|---|---|---|---|
| 10 | Ashby | Sterile | Redistilled | 1.22 | ..... |
| 11 | Ashby | Inoc. | Redistilled | Lost | ..... |
| 12 | Ashby | Inoc. | Redistilled | 4.73 | 3.51 |
| 19 | Beijerinck | Sterile | Redistilled | 1.05 | ..... |
| 20 | Beijerinck | Inoc. | Redistilled | 3.35 | 2.30 |
| 21 | Beijerinck | Inoc. | Redistilled | 2.88 | 1.83 |
| 22 | Beijerinck | Sterile | Tap | 1.13 | ..... |
| 23 | Beijerinck | Inoc. | Tap | Lost | ..... |
| 24 | Beijerinck | Inoc. | Tap | 7.70 | 6.57 |

[1] In the original Beijerinck medium dipotassium phosphate is used, and, of course, no neutralization to phenolphthalein is necessary.

It seems that the Beijerinck solution prepared with tap water (i. e., the true Beijerinck solution) is superior to Ashby's solution when equal amounts of colloidal $Fe_2O_3$ are added to each. The loss of culture 23 is very unfortunate, but the notes describe cultures 23 and 24 as rapid and vigorous growths, as indicated first by turbidity, then zoögloea formation at junction of liquid surface with walls of glass flask, and later by the formation of a finely flocculent precipitate at the bottom of the flask.

The growth in cultures 20 and 21, while far below that in the regular Beijerinck solution, is really greater than we had reason to expect. This indicates that needs of the organism for mineral nutrients, aside from sodium, potassium, phosphorus, and iron, must be very low indeed and were partly covered by the impurities carried by the constituents of the medium.

It is interesting to note in this connection that it is more or less generally recognized by bacteriologists that tap water is superior to distilled water for the preparation of regular media. The objection to its universal use is its inconstancy of composition and the consequent varying results which attend its use in different laboratories or even in the same laboratory at different times. Now, soil biologists recognize the significance of this factor and moreover that in many cases soil extract is superior to tap water. In the case of *Azotobacter*, for instance, it is quite generally known that Ashby's soil extract medium is superior to the regular Ashby medium in which distilled water is used, and to that end we used Ashby's soil extract agar to some extent in the propagation of our stock culture. It is also universally recognized by chemists, and to a less extent by biologists, that distilled water is not free from dissolved substances. Inorganic salts are carried over mechanically entrained in the vapor, and volatile organic compounds are with difficulty completely destroyed. It seemed worth while in this work to remeasure the magnitude of the differences resulting from the use of tap, distilled, and redistilled water. The stock laboratory distilled water was prepared by an electric still with a preheating device, and was

stored in a tin-lined copper tank and from here distributed to the laboratories by a system of block tin tubes. The redistilled water was prepared by distilling this water over acid permanganate. The connection between the block tin condenser and the distilling flask was made by wadding with absorbent cotton. The results obtained are reported in table III below. The data on redistilled water are, it will be noted, those reported before in table I as cultures 1, 2, and 3.

TABLE III

INFLUENCE OF DIFFERENT WATERS USED IN PREPARATION OF MODIFIED ASHBY'S NUTRIENT SOLUTION

| No. | Water used | Treatment | N found (mgs.) | N fixed (mgs.) |
|---|---|---|---|---|
| 1 | Double distilled | Sterile | 1.31 | ..... |
| 2 | Double distilled | Inoc. | 2.59 | 1.28 |
| 3 | Double distilled | Inoc. | 1.98 | 0.67 |
| 13 | Stock distilled | Sterile | 1.03 | ..... |
| 14 | Stock distilled | Inoc. | 3.58 | 2.55 |
| 15 | Stock distilled | Inoc. | 2.78 | 1.75 |
| 16 | Tap | Sterile | 1.26 | ..... |
| 17 | Tap | Inoc. | 3.49 | 2.23 |
| 18 | Tap | Inoc. | 4.45 | 3.19 |

The differences observed are not wide but serve to show the order of magnitude of the effect produced by different waters, and also illustrate the fact that something is lacking in Ashby's solution.

As mentioned above, very great difficulty was experienced in digesting the cultures according to the Kjeldahl-Gunning method. We decided next to see to what extent this difficulty could be overcome by using a very much smaller amount of material, a principle that finds extended use in the "micro" methods of biological chemistry. Accordingly, 10-cc. portions of Ashby's solution were placed in 60-cc. Erlenmeyer flasks. The medium was made as above except that tap water was used. Calcium carbonate was omitted in one-half the culture flasks, since, as mentioned above, it seemed to exert a quite marked precipitating effect on the colloidal ferric oxide which was added in the amounts of 0.01, 0.05, and .1 mg. per 10 cc. of culture solution. The flasks were inoculated with a spiral

of a suspension prepared as described above from a 24-hour-old streak of the $F_2$ generation on Ashby's soil extract agar. The "micro" cultures were kept in an incubator at 30–31° C. for two weeks and then analyzed for total nitrogen according to the procedure described above except that 15 cc. concentrated $H_2SO_4$, 7 gms. $Na_2SO_4$, and 2 cc. 10 per cent $CuSO_4$ were used for each digestion. As the amount of mannite was only one-tenth that in the previously described experiments, digestion was completed in much less time. It was, however, not to be designated as rapid or free from the annoyance of foaming. In fact, from 3 to 5 hours were required for the digestion mixtures to clear, and some cultures were lost as a result of foaming out of the digestion flasks.

The results are reported in table IV. The data on fixation are computed to mgs. per 100 cc., i. e., 10 times the amount actually observed.

TABLE IV

FIXATION OF NITROGEN BY AZOTOBACTER CHROOCOCCUM IN MODIFIED ASHBY'S SOLUTION

| No. | Addition to culture medium | Treatment | No $CaCO_3$ | | $CaCO_3$ added | |
|---|---|---|---|---|---|---|
| | | | N found per culture (mgs.) | N fixed per 100 cc. (mgs.) | N found per culture (mgs.) | N fixed per 100 cc. (mgs.) |
| 25 | Control | Sterile | .360 | ..... | .439 | ..... |
| 26 | Control | Inoc. | .544 | 1.84 | .660 | 2.21 |
| 27 | Control | Inoc. | .487 | 1.27 | .660 | 2.21 |
| 28 | 0.01 mg. $Fe_2O_3$ | Sterile | .329 | ..... | .453 | ..... |
| 29 | 0.01 mg. $Fe_2O_3$ | Inoc. | .646 | 3.17 | 1.357 | 9.04 |
| 30 | 0.01 mg. $Fe_2O_3$ | Inoc. | .601 | 2.72 | .850 | 3.97 |
| 31 | Control | Sterile | .227 | ..... | .190 | ..... |
| 32 | Control | Inoc. | .351 | 1.24 | .422 | 2.32 |
| 33 | Control | Inoc. | Lost | ..... | .448 | 2.58 |
| 34 | 0.05 mg. $Fe_2O_3$ | Sterile | Lost | ..... | .133 | ..... |
| 35 | 0.05 mg. $Fe_2O_3$ | Inoc. | .756 | 5.29* | .955 | 8.22 |
| 36 | 0.05 mg. $Fe_2O_3$ | Inoc. | .674 | 4.47* | .949 | 8.16 |
| 37 | Control | Sterile | .196 | ..... | .234 | ..... |
| 38 | Control | Inoc. | .334 | 1.38 | Lost | ..... |
| 39 | Control | Inoc. | .326 | 1.30 | .521 | 2.87 |
| 40 | 0.10 mg. $Fe_2O_3$ | Sterile | .210 | ..... | .215 | ..... |
| 41 | 0.10 mg. $Fe_2O_3$ | Inoc. | .734 | 5.24 | .997 | 7.82 |
| 42 | 0.10 mg. $Fe_2O_3$ | Inoc. | .615 | 4.05 | .906 | 6.91 |

* Computed from No. 31 as blank.

The application of "micro" technique to the problem at hand did not seem especially promising, as the nitrogen determinations were still accompanied with considerable difficulty, and since the error of the analysis was almost as great, the final value computed to mgs. N per 100 cc. contains an appreciably greater error. This point is discussed again below.

Although the data are somewhat erratic, it seems permissible to conclude that the addition of colloidal iron to Ashby's solution produces a beneficial effect and also that calcium carbonate has a beneficial action even though the reaction of the culture medium is carefully adjusted beforehand, and even though it appears to cause a greater flocking out of the colloidal ferric oxide.

It seems that we can conclude with reasonable safety from the above admittedly crude results that in a general way the work of Remy and Rösing has been confirmed. The beneficial results obtained by the addition of colloidal ferric oxide to culture solutions are much less marked than those of Remy and Rösing, yet there seems to be no reasonable doubt that such action is well worth further study, particularly in the line of the rôle of the colloidal ferric oxide. Before such a study can be carried on advantageously it is necessary to make a decided improvement in experimental methods. This point will now be considered.

## IMPROVEMENT IN METHODS

One of the first points to be considered in the improvement of experimental methods was that of a suitable method of sugar determination. The exact measurement of the energy consumption in cultures of *Azotobacter* is worthy of much more study than it has received. Many workers have made computations on the amount of mannite or carbohydrate added to the culture medium, disregarding the residual amount of energy-supplying material. The exact determination of mannite is not feasible, and the determinations that have been made of dextrose have been accomplished with the

use of crude and cumbersome methods that have not especially invited further work in this direction.

In the experiments reported above 10-cc. and 100-cc. portions of culture media were used. The former did not render the Kjeldahl digestion sufficiently easy, whereas the others were so large that with the containers available it was almost impossible to keep the culture solution shallow enough to permit proper growth. The proper line of improvement seemed to be, therefore, to improve conditions as to methods of digestion for cultures intermediate in size between the above extremes, i. e., having a volume of 25–50 cc. The methods of distilling and recovering the ammonia were not wholly satisfactory, and some studies of refinement of distillation methods were made.

Another source of error or annoyance in the above experiments was the matter of a uniform method of inoculating a series of flask cultures. Of course, inoculating directly from an agar slant with a platinum loop is open to considerable objection on the ground of lack of uniformity. Inoculations should preferably be as small as possible where quantitative chemical determinations are to be made on the culture, and to this end attempts were made to inoculate the culture flasks with either one cc. or a spiral of a suspension of 1 spiral of agar slant growth in 50 cc. of sterile water. Results were uncertain, in fact almost wholly negative. The point seemed therefore worthy of further study.

The work on improvements in methods (1) of sugar determination, (2) of nitrogen methods, and (3) of inoculation, will now be considered seriatim.

### DETERMINATION OF SUGAR IN AZOTOBACTER CULTURES

For the determination of dextrose in cultures of *Azotobacter* it seemed to us to be worth while to attempt to adopt some of the more modern methods to the problem in hand. The method of Shaffer ('14) appeared most promising from the standpoint of ease of manipulation and accuracy of results, and, with only very minor modifications, it proved to be applicable to cultures of *Azotobacter*. The principle of the

method is that proteins are removed by the Michaelis-Rona colloidal iron precipitation, and the centrifuge used for clarifying the solution and recovery of the cuprous oxide, which is then determined by Bertrand's method.

In our first experiments dextrose was determined on one culture and nitrogen on its duplicate; later the procedure was modified so that sugar and nitrogen were determined on the same culture with reasonable accuracy. The procedure finally adopted follows: The culture medium is acidified with N/2 HCl and warmed till mineral salts are in solution and the proteins dispersed to an opalescent solution. After cooling the material is transferred to a 100-cc. or a 250-cc. volumetric flask, depending on the size of the culture, and made to the mark. An aliquot of this suspension is transferred to a 100-cc. volumetric flask, the volume made to approximately 75 cc., and a pinch of sodium acetate added to reduce the hydrogen ion concentration. Five cc. of Merck's colloidal iron are then added, the suspension well mixed, and approximately 0.2 gm. $Na_2SO_4$ added, and water added to the mark. The suspension is again well mixed, poured into a 100-cc. centrifuge tube, and centrifuged for 15 minutes. Duplicate 20-cc. portions of the clear supernatant liquid are then transferred to 50-cc. centrifuge tubes. The procedure from this point on is the same as that outlined by Shaffer.

The method of Shaffer is really a "micro" method proposed for the determination of sugar in blood, where only small samples of a tissue low in sugar can be analyzed. The conditions worked out by Shaffer cover naturally a comparatively narrow range of dextrose amounts; hence in working with cultures of *Azotobacter* which contain, in the controls at least, very large amounts of sugar, it is easily possible to draw off in aliquoting a too large amount of dextrose in solution. On the other hand, it is just as easy to remove an aliquot so small that the error of the analysis is multiplied by a too large factor in computing the amount of sugar in the portion of culture medium under examination. The latter error was made in some of our determinations and probably accounts for the results indicated below as being of questionable value.

We obtained most satisfactory results by aliquoting, so that the final amount reduced in the centrifuge tube corresponded to 2 cc. of 2 per cent dextrose nutrient solution.

Nitrogen is determined on the remainder of the culture solution and computed to the total amount of the original culture.

## NITROGEN METHODS

*Distillation.*—The distillation apparatus used above, which had proved very satisfactory in the Wooster laboratory for distillations from weakly alkaline solutions, did not prove entirely satisfactory for distillations from the strongly alkaline solutions used in the Kjeldahl method. A very slight escape of ammonia was detected from the receiver flask by means of a second receiver flask. The introduction of a cooled condenser was therefore necessary. At the same time we made some experiments on an apparatus without rubber connections. This device is shown in fig. 1. The mouth of the Pyrex flask *A* was flared slightly and ground to fit the head *B* which was sealed on to the condenser tube *C*, the latter being provided with the water jacket *D* 13 inches in length and terminating in the perforated bulb *E*.

The difficulty attending the use of a cooled condenser is that complete transfer of the ammonia requires sufficient distillation to increase the volume in the receiver to a point where accurate titrations with N/50 solutions are interferred with. This difficulty is overcome to a considerable extent by the use of the principle employed by Benedict[1], i. e., distilling into the cooled condenser for 15 or 20 minutes, then draining the condenser and completing the distillation. Using the above apparatus and distilling slowly for 20 minutes through a cooled condenser, then draining and continuing the distillation for 20 minutes longer, quantitative transfer of the ammonia was effected and the volume of the receiver kept fairly low. As zinc was used to prevent bumping and as no provision for scrubbing was included in the apparatus, a second distillation over N/10 NaOH was necessary. The magnitude

[1] Benedict, F. G. The distillation of ammonia in the determination of nitrogen. Am. Chem. Soc., Jour. **22** : 259–263. *f. 1.* 1900.

of the error from the mechanical carrying-over of the alkali may be seen in table VI where "first" and "second" distillations are recorded. This apparatus was only moderately satisfactory. The volume of the receiver flasks varied from 130 to 150 cc., whereas a range of 100 to 115 cc. would have

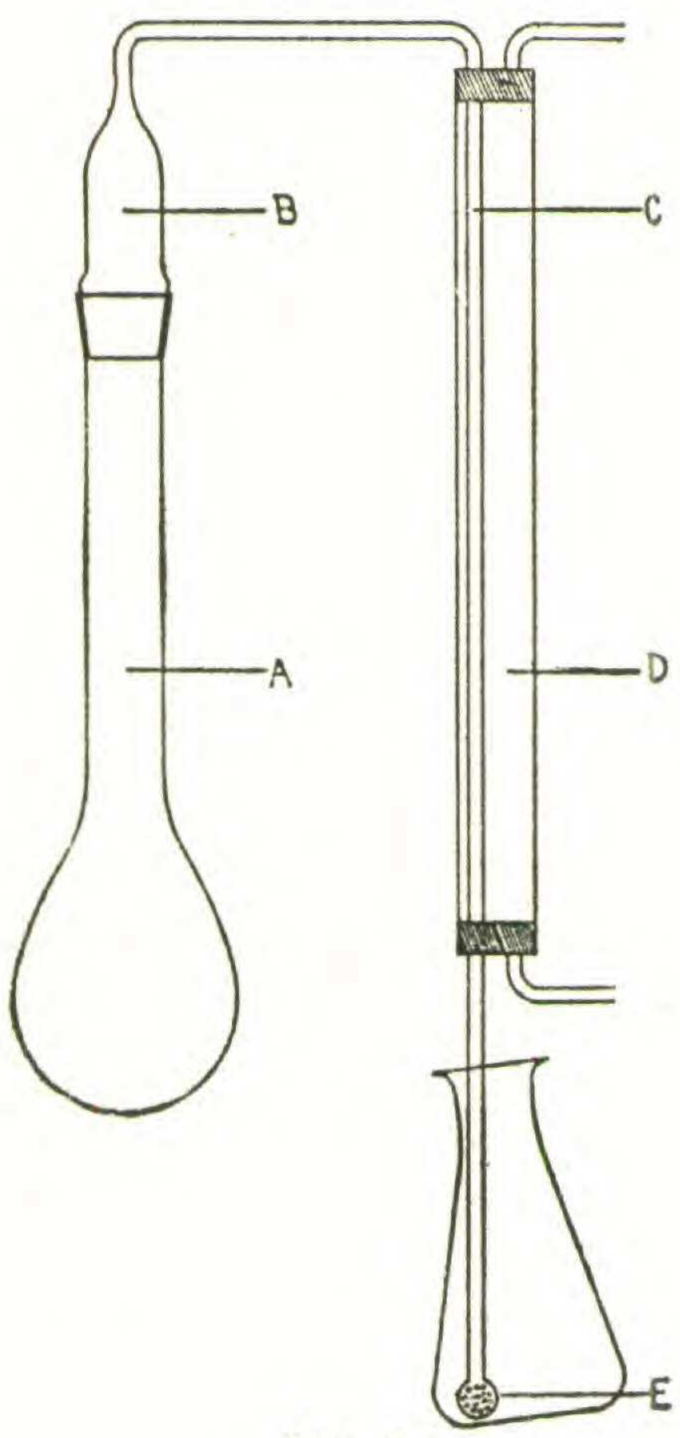


Fig. 1

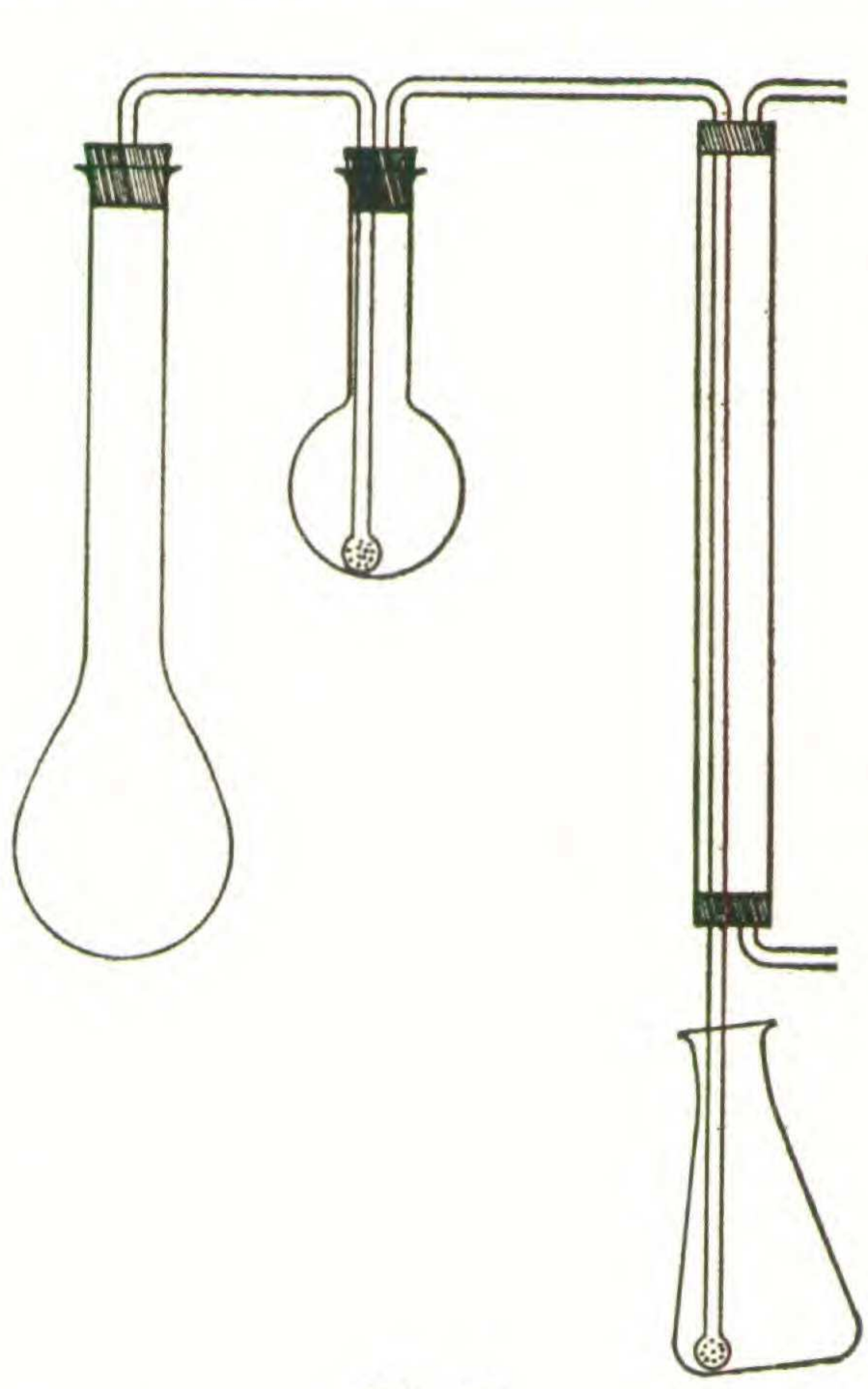

Fig. 2

Nitrogen-distilling apparatus.

been more satisfactory. Moreover, the error incident to redistillation detracts from the greater accuracy resulting from the elimination of rubber stoppers. The final apparatus adopted is that shown in fig. 2, which is self-explanatory. Pyrex glass was used throughout except for condenser jackets. Distillation was carried on slowly for 20 minutes through a cooled condenser and 20 minutes after draining the condenser. It was found by a series of distillations on ammonium sulphate solutions that quantitative recovery was effected by this procedure, and the volume of the receiver flasks as a rule remained below 115 cc. at the end of the distillation period. This

apparatus was used throughout the "Final" experiments reported below.

From this point the matter of a distillation apparatus has been developed as a separate problem and is reported in a following paper (Allen and Davisson, '19).

*Kjeldahl digestions.*—As stated above, the foaming accompanying solutions high in mannite was most troublesome, and it was realized that unless some improvement could be devised future progress would be almost blocked. Varying conditions with respect to catalysts, i. e., using different amounts of mercury, copper sulphate, and metallic copper, did not give any appreciable aid. The foaming appeared to be due to separation at the outset of a large amount of carbon or highly carbonaceous material which is very resistant to decomposition in boiling sulphuric acid. It occurred to us that the high temperature imparted to the digestion mixtures by the sodium (or potassium sulphate) caused a heavier deposit of this material than would otherwise be the case. Experiment proved the correctness of this suggestion, and it was found that by carrying on the digestion for 20 to 25 minutes with sulphuric acid and copper sulphate alone, then adding the sodium sulphate, the danger from foaming was slight and the digestion mixture containing 2 gms. mannite cleared in from 60 to 85 minutes.

The empirical experiments with catalysts were then repeated, with the result that copper sulphate alone seemed to be the most desirable agent. Judging by the time required for the clearing of the mixtures, mercury adds but little to the effect of $CuSO_4$ and possesses the disadvantage, of course, that $Na_2S$ must be added to the alkali. The above experiments were made with $CuSO_4$,[1] 2.0 gms., Hg, 3 drops, and Cu, 0.5 gm. It was noted that the above amount of $CuSO_4$ was very efficient in inhibiting foaming. Hibbard[2] found that large amounts of $CuSO_4$ were associated with incomplete recovery of $NH_3$ by distillation. To determine the minimum amount of $CuSO_4$ required to prevent foaming varying amounts of this

[1] All references to copper sulphate refer to the hydrate $CuSO_4 .5 H_2O$.

[2] Hibbard, P. L. Notes on the determination of nitrogen by the Kjeldahl method. Jour. Ind. and Eng. Chem. **2**: 463–466. 1910.

salt were added to the digestion flasks containing 2 gms. mannite and 30 cc. of conc. $H_2SO_4$. After digestion had proceeded for 20 minutes, 10 gms. of $Na_2SO_4$ (anhydrous) were introduced into each flask. The time for the solutions to become clear bluish green and the degree of foaming were recorded. The results appear in table v.

TABLE V

EFFECT OF VARYING AMOUNTS OF COPPER SULPHATE

| No. | Amount $CuSO_4$ (gms.) | Time of clearing (minutes) | Order | Foaming |
|---|---|---|---|---|
| 1 | 0.1 | 73 | 3 | Decided |
| 2 | 0.2 | 68 | 2 | Decided |
| 3 | 0.3 | 78 | 4 | Decided |
| 4 | 0.4 | 80 | 5 | Moderate |
| 5 | 0.5 | 63 | 1 | None |

The differences in regard to time are of minor significance. The differences in regard to foaming are important and indicate that as measured by this standard less than 0.5 gm. of $CuSO_4$ should not be used. The digestion procedure used in the "Final Experiments" was as follows: The sample is digested for 20 or 25 minutes with 30 cc. of $H_2SO_4$ and 0.5 gm. $CuSO_4$. Ten grams anhydrous $Na_2SO_4$ were then added and the digestion completed. The above results were confirmed repeatedly with the slight difference that the digestion of nutrient mannite or dextrose solutions required a trifle longer than the pure mannite, this probably for the reason that the salts of the solution were slightly inhibitory in action. As a general average the mixtures cleared in $1\frac{1}{2}$ hours; in all cases the digestion was continued over a low flame for $1\frac{1}{2}$ hours after clearing.

## METHODS OF INOCULATION

Two methods of inoculation were used, and 10 cultures seeded by each method were incubated and analyzed. By the one method, a spiral of growth from an agar slant was transferred to 10 cc. of Ashby's solution (plus $CaCO_3$) contained in a 250-cc. Erlenmeyer flask, and the culture maintained on the shaker for 24 hours in the warm room at 28–30° C. One spiral

of this suspension was then used as the inoculum. This procedure was designated as method *A*.

In the other method of inoculation a spiral of the growth on an agar slant was introduced into a regular 10-cc. water blank, well shaken, and a spiral of this used at once as the inoculum. This procedure was designated as method *B*.

Twenty 250-cc. flasks were prepared, each containing 20 cc. of Ashby's solution (plus $CaCO_3$). Ten of these were inoculated by method *A* and 10 by method *B*. For preparing the suspension in the former, the growth from a 72-hour Ashby soil extract agar slant of $F'_4$ generation was used, while for method *B*, material from the same slant 76 hours old was used; that is, all flasks were seeded on the same date. After inoculation the cultures were incubated on the shaking machine[1] in the warm room which remained at 28–30° C. except one night when it dropped to 22° C. for several hours. Cultures inoculated according to method *A* showed a distinct and uniform turbidity on the third day, while those inoculated according to method *B* showed no visible growths until the fourth day, and these were less distinct and less uniform than those observed in the other set at 3 days. An incubation period of only 5 days was used, as it was believed that a short period

TABLE VI
COMPARISON OF METHODS OF INOCULATION

| Culture no. | Inoculated according to: | | | |
|---|---|---|---|---|
| | Method A | | Method B | |
| | 1st dist. (mgs. N) | 2nd dist. (mgs. N) | 1st dist. (mgs. N) | 2nd dist. (mgs. N) |
| 1 | 2.26 | 1.31 | 2.41 | 0.97 |
| 2 | 1.59 | 1.26 | 2.44 | 0.93 |
| 3 | 2.01 | 1.14 | 1.18 | 1.10 |
| 4 | 1.64 | 1.10 | 1.97 | 0.92 |
| 5 | 6.08 | 1.14 | Lost* | 0.81 |
| 6 | 4.05 | 1.51 | 1.10 | 0.96 |
| 7 | 2.31 | 1.03 | 1.71 | 0.78 |
| 8 | 3.34 | 1.12 | 1.11 | 0.97 |
| 9 | 3.07 | 1.21 | 1.81 | 0.83 |
| 10 | 1.74 | 1.03 | 1.67 | 1.04 |
| Ave. | ..... | 1.185 | ..... | 0.93 |

* Visible amount of alkali carried over mechanically.

[1] At the time of setting up this experiment, the mechanical difficulties attending the construction of a satisfactory and reliable rotary shaker had not been overcome, so an ordinary laboratory shaker was geared down so as to tilt the flasks back and forth at a rate of 3 complete excursions each 2 minutes.

would reveal irregularities in the method of inoculation better than would a longer period. The results reported as mgs. N per culture are shown in table VI.

Method *A*, in which the suspension was incubated 24 hours on a shaking machine, gave slightly higher results, yet growth was certain and reasonably uniform in those inoculated according to method *B*. It is rather difficult to decide between these two methods on the basis of the above experiment. In subsequent work method *A* was favored, but if for any reason it was undesirable to delay the experiment 24 hours method *B* was used. The significant thing is that growth took place in every flask seeded. The difficulty encountered in the "orientation experiments" was probably due to the fact that the suspensions were too dilute. No more difficulty was experienced in obtaining growth from the heavy suspensions used. However, some rather surprising failures of growths on plates from dilutions from the suspensions were observed. The point seemed to be that in working with *Azotobacter* heavier suspensions must be used than are needed with most other bacteria. This is possibly to be explained by their heavy slime production.

## FINAL EXPERIMENTS

The few experiments which we were able to carry out after revising our methods were designed to see how far the facts would fit the hypothesis suggested above; that is, to what extent certain variations with regard to the presence of a second phase in the nutrient solution would affect the growth and development of the microörganism under study. Experiments were conducted on the following points: (1) removal of the solid phase; (2) restoring of the solid phase; (3) homogenous nutrient solutions; and (4) the action of protective colloids designed to partially or wholly prevent the flocking out of the phosphate precipitate. These experiments will now be considered in the order named.

### REMOVAL OF SOLID PHASE

For the study of this point Ashby tap water dextrose[1] me-

[1] Whenever dextrose was used the culture medium was sterilized by the intermittent method.

dium was used. One portion was boiled up with calcium carbonate and then filtered through a folded filter. The filtrate was perfectly clear. Fifty-cc. portions were pipetted into 700-cc. "Nonsol" Erlenmeyer flasks, each containing a pinch of calcium carbonate. A parallel series was set up, using the unheated and unfiltered medium. After sterilization alternate pairs of flasks in each series were inoculated with a spiral of a 24-hour shaker culture of *Azotobacter* prepared by inoculating 10 cc. of Ashby's mannite solution in a 250-cc. Erlenmeyer flask with a spiral of a 72-hour growth of $F_{10}$ on Ashby soil extract agar.

One-half of each series was placed on the rotary shaker and one-half on the shelf near by. The whole experiment was carried out in the warm room, the temperature of which during this particular period was very erratic. The first two days

TABLE VII

SERIES A—SOLID PHASE PRESENT

| No. | Treatment | Condition at close | Nitrogen (mgs.) |
|---|---|---|---|
| | | Shaker | |
| 1 | Check | Clear | 0.62 |
| 2 | Check | Clear | 0.59 |
| 3 | Inoc. | Strong turbidity, no floccules | 5.10 |
| 4 | Inoc. | Strong turbidity, floccules and slight pigment | 5.40 |
| | | | Residual sugar (mgs.) |
| 5 | Check | Clear | 848. |
| 6 | Check | Clear | 839. |
| 7 | Inoc. | Same as No. 4 | 0.0 |
| 8 | Inoc. | Same as No. 4 | 0.0 |
| | | Shelf | |
| | | | Nitrogen (mgs.) |
| 9 | Check | Clear | 0.72 |
| 10 | Check | Clear | 0.65 |
| 11 | Inoc. | Good turbidity, some floccules | 3.34 |
| 12 | Inoc. | Good turbidity, some floccules | 2.97 |
| | | | Residual sugar (mgs.) |
| 13 | Check | Clear | 820. |
| 14 | Check | Clear | 825. |
| 15 | Inoc. | Same as Nos. 11 and 12 | 449. |
| 16 | Inoc. | Same as Nos. 11 and 12 | 559. |

the temperature was 23–25° C., then 28–30° for 3 days, while for the last 5 days of the 10-day incubation period it was 35–37°. The rotating machine[1] was revolved once in 24 seconds. After 10 days one-half of the flasks of the shaker set and of the shelf set were subjected to nitrogen analyses, and the other half to sugar determinations. The results appear in tables VII and VIII.

TABLE VIII

SERIES B—SOLID PHASE REMOVED

| No. | Treatment | Condition at close | Nitrogen (mgs.) |
|---|---|---|---|
| | | Shaker | |
| 17 | Check | Clear | 0.42 |
| 18 | Check | Clear | 0.42 |
| 19 | Inoc. | Clear | 0.41 |
| 20 | Inoc. | Faint turbidity | 0.68 |
| | | | Residual sugar (mgs.) |
| 21 | Check | Clear | ...* |
| 22 | Check | Clear | ...* |
| 23 | Inoc. | Clear | 881. |
| 24 | Inoc. | Faint turbidity | 600. |
| | | Shelf | |
| | | | Nitrogen (mgs.) |
| 25 | Check | Clear | 0.42 |
| 26 | Check | Clear | Lost |
| 27 | Inoc. | Faint turbidity | 1.11 |
| 28 | Inoc. | Faint turbidity | 1.75 |
| | | | Residual sugar (mgs.) |
| 29 | Check | Clear | 959. |
| 30 | Check | Clear | 945. |
| 31 | Inoc. | Clear | 933. |
| 32 | Inoc. | Faint turbidity | 914. |

* By mistake these cultures were subjected to nitrogen analyses. Nos. 21 and 22 contained 0.393 and 0.418 mgs. N, respectively.

This experiment brings out very clearly the effect of removing the precipitated phosphates from Ashby's solution. The medium from which this precipitate has been removed is very poorly suited to the support of this microörganism. It seems that the shaker is detrimental with this medium, while it is distinctly beneficial when the precipitate of phosphates is

[1] Constructed similar to the one used by Bonazzi, *loc. cit.*

present. The variation in the growths in different flasks shows the undesirability of making a sugar determination on one flask and a nitrogen determination on its duplicate. The sugar determinations above are comparatively inaccurate, owing to the fact that only $\frac{1}{20}$ of the solution was taken for analysis, hence computations of nitrogen fixed per gram of dextrose consumed have not been made.

### RESTORATION OF SOLID PHASE

An experiment was next conducted on adding the solid substances, $CaCO_3$ and $CA_3\ (PO_4)_2$, alone and in combination, to filtered (i. e., filtered subsequent to heating to boiling in the presence of $CaCO_3$) Ashby's tap water solution. The culture medium was therefore directly comparable to that used in obtaining the data reported in tables VII and VIII. Twenty-five-cc. portions of this perfectly clear solution were pipetted into 700-cc. flasks and a drop of 0.5 ferric chloride added to each. Calcium carbonate and calcium phosphate were added to certain of the flasks according to the plan shown in table IX, which contains also the results of the experiment. The flasks were inoculated at the same time and from the same suspension as used in the previous experiment. All flasks were incubated on the rotating machine for 10 days.

TABLE IX

| No. | Material added to culture medium | Treatment | Residual glucose (mgs.) | Nitrogen (mgs.) |
|---|---|---|---|---|
| 45 | None | Inoc. | 294.1 | ... |
| 46 | None | Inoc. | 215.4 | ... |
| 47 | $CaCO_3$ | Inoc. | 181.7 | ... |
| 48 | $CaCO_3$ | Inoc. | 448.8 | ... |
| 49 | $Ca_3\ (PO_4)_2$ | Inoc. | 409.0 | 0.45 |
| 50 | $Ca_3\ (PO_4)_2$ | Inoc. | 562.0* | 0.44 |
| 51 | $CaCO_3$, $Ca_3(PO_4)_2$ | Inoc. | 00.0 | 2.16 |
| 52 | $CaCO_3$, $Ca_3(PO_4)_2$ | Inoc. | 00.0 | 2.14 |
| 53 | $CaCO_3$, $Ca_3(PO_4)_2$ | Sterile | 448.0 | 0.19 |
| 54 | $CaCO_3$, $Ca_3(PO_4)_2$ | Sterile | 450.5 | 0.16 |

* Probably an analytical error.

Owing to an accident in the introduction of the alkali into the Kjeldahl flasks to the set of 4 cultures, 45–48, these nitrogen determinations were lost. In spite of these irregularities the experiment shows undoubted benefit resulting from the addition of tricalcium phosphate, the same material filtered off in experiment 1 above. It is interesting to note that only when both the carbonate and the phosphate of calcium were added was good growth obtained.

### MEDIA WHICH FORMED NO PRECIPITATE

*Medium of Löhnis and Smith.*—Löhnis and Smith ('16, p. 686) state that a medium of the following composition is excellent for supporting the growth of *Azotobacter* and remains perfectly clear:

| | | |
|---|---|---|
| Dextrose | 20 | gms. |
| Dipotassium phosphate[1] | 0.2 | gm. |
| Sodium chloride | 0.2 | gm. |
| Magnesium sulphate | 0.2 | gm. |
| Calcium sulphate | 0.1 | gm. |
| 10% ferric chloride | 2 | drops |
| Distilled water | 1000 | cc. |

This medium is essentially Ashby's solution, hence we felt sure it would yield a precipitate. When the above materials were dissolved in the cold the solution was almost but not quite clear. On heating to boiling a slight flocculent precipitate formed. After the solution had cooled this precipitate was filtered off and 25-cc. portions of the perfectly clear filtrate pipetted into 1000-cc. Erlenmeyer flasks. Two series of flasks of 6 each were prepared, the one series receiving a pinch of calcium carbonate per flask, the other not. The method of inoculation was the same as that used in the two previous experiments, the same suspension being used. Two flasks of each series were placed on the rotator, and the remaining ones on the shelf near by. The incubation conditions were the same as in previous experiments. The results are given in table x.

[1] Löhnis and Smith used monopotassium phosphate neutralized to phenolphthalein with sodium hydroxide.

TABLE X

AZOTOBACTER DEVELOPMENT IN FILTERED LÖHNIS AND SMITH'S MEDIUM

| | No. | Treatment | Glucose (mgs.) | Nitrogen (mgs.) |
|---|---|---|---|---|
| Shelf | 55 | Check | 464. | .42 |
| | 56 | Check | 452. | .34 |
| | 57 | Inoc. | 486. | .41 |
| | 58 | Inoc. | 251. | .34 |
| Shaker | 59 | Inoc. | 530. | .39 |
| | 60 | Inoc. | 524. | .37 |
| Same conditions except $CaCO_3$ added | | | | |
| Shelf | 61 | Check | 530. | .32 |
| | 62 | Check | 516. | .30 |
| | 63 | Inoc. | 339. | .75 |
| | 64 | Inoc. | 385. | .90 |
| Shaker | 65 | Inoc. | Lost but made abundant growth | ... |
| | 66 | Inoc. | 328. | .88 |

The clear filtered medium is very poor for the growth of *Azotobacter;* indeed there is no evidence that growth took place. When calcium carbonate is added growth is better. The fact that growth takes place in this filtered medium, whereas it failed in our first experiment, is probably due to all phosphates not being removed by the method of precipitation in this experiment, whereas they were in the first one.

*Glycerolphosphate medium.*—In order to prepare a medium which would remain clear and from which the phosphates would not be precipitated by heating in presence of $CaCO_3$, an organic phosphate was used. Calcium glycerolphosphate seemed to be the most promising, since it is soluble in water and does not form a precipitate with any of the salts used in Ashby's solution. Twenty-four hundredths gm. of this salt carries essentially the same amount of phosphorus as does .2 gm. $K_2HPO_4$ and a little more calcium than does .1 gm. $CaSO_4 . 2H_2O$. Hence this amount of calcium glycerolphosphate added to the medium supplies as much phosphorus and calcium to the culture medium as is contained in Ashby's solution. Since the molar weights of $K_2HPO_4$ and $K_2SO_4$ are practically equal, .2 gm. of $K_2SO_4$ will carry the same amount

of potassium as .2 gm. $K_2HPO_4$. The following medium was therefore prepared:

| | | |
|---|---|---|
| Dextrose | 20 | gms. |
| Calcium glycerolphosphate | 0.2 | gm. |
| Magnesium sulphate | 0.2 | gm. |
| Sodium chloride | 0.2 | gm. |
| Potassium sulphate | 0.2 | gm. |
| Distilled water | 1000 | cc. |
| 10% ferric chloride | 3 | drops |

No precipitate formed on heating the medium. It was filtered, however, to remove the few particles of foreign material which most likely were introduced in the dextrose. The perfectly clear medium then was found to give a $P_H$ value of about 6.7 as measured with the aid of standards and buffer solutions recommended by Clark and Lubs ('17). Duplicate 5-cc. portions were titrated with N/50 alkali and phenolphthalein and the computed amount (9.00 cc.) of N/50 alkali necessary to bring an aliquot of the medium to $P_H$ 8. After the addition of this amount of alkali the $P_H$ value was found to be a trifle less than 8.

Fifty-cc. portions of this adjusted medium were then pipetted into each of twelve 700-cc. "Nonsol" Erlenmeyers. To 6 of these was then added a pinch of calcium carbonate. After sterilization by the intermittent method the cultures were incubated for 7 days at 28–30° C. The plan of the experiment is shown in table XI. The inoculum was from a 24-hour 10-cc. mannite shaker culture prepared by heavy inoculation from $F_{17}$, 7 days old. One spiral of this culture was used in seeding each flask. The results appear in table XI.

The results indicate that the phosphorus in glycerolphosphates is to some extent available for the growth of *Azotobacter,* although it is barely possible that the glycerolphosphate may have hydrolyzed in the faintly alkaline solutions during sterilization. The medium, although adjusted in reaction to practically $P_H$ 8, was not suitable for growth unless $CaCO_3$ was added, practically no growth taking place in the absence of $CaCO_3$. The rotator proved beneficial in the presence of calcium carbonate.

TABLE XI

AZOTOBACTER DEVELOPMENT IN THE PRESENCE OF GLYCEROLPHOSPHATE

| | No. | Treatment | Base added | Cont. of culture at end | |
|---|---|---|---|---|---|
| | | | | Sugar (mgs.) | Nitrogen (mgs.) |
| Shelf | 67 | Check | 0 | 910. | 0.16 |
| | 68 | Check | 0 | 914. | 0.16 |
| | 69 | Inoc. | 0 | 913. | 0.10 |
| | 70 | Inoc. | 0 | 912. | 0.13 |
| Shaker | 71 | Inoc. | 0 | 908. | 0.08 |
| | 72 | Inoc. | 0 | 908. | 0.10 |
| Shelf | 73* | Check | $CaCO_3$ | 901. | 0.09 |
| | 74* | Check | $CaCO_3$ | 904. | 0.22 |
| | 75 | Inoc. | $CaCO_3$ | 848. | 0.67 |
| | 76 | Inoc. | $CaCO_3$ | 820. | 0.99 |
| Shaker | 77 | Inoc. | $CaCO_3$ | 275. | 1.88 |
| | 78 | Inoc. | $CaCO_3$ | 268. | 2.05 |

* 500-cc. flasks.

ACTION OF PROTECTIVE COLLOIDS

If now one of the effects of mechanical agitation is to hasten the solubility of phosphates, it ought to be possible to replace this action in part by the use of protective colloids; that is, the colloid, by preventing the complete flocking out of these compounds, would cause a greater surface to be exposed to the action of the solvent. Agar naturally suggested itself as a possible protective colloid, and its function as such was studied in two ways: (a) in solid media, and (b) in filtered and non-filtered liquid media.

*Solid nutrient agars.*—Two agars were prepared from purest chemicals obtainable and redistilled water, and these compared with the regular nutrient agar. In the case of the one agar the phosphates were allowed to precipitate before the agar was added. In the case of the second medium one half the agar was added to a solution containing the calcium and magnesium salts, the other half to a solution containing the phosphate. The exact procedures were as follows:

Agar I (phosphates allowed to precipitate before the addition of agar).

Solution A

| | | |
|---|---|---|
| Mannite | 20 | gms. |
| Dipotassium phosphate | 0.2 | gm. |
| Double distilled water | 500 | cc. |

Solution B

| | | |
|---|---|---|
| Magnesium sulphate | 0.2 | gm. |
| Sodium chloride | 0.2 | gm. |
| Calcium sulphate | 0.1 | gm. |
| Double distilled water | 500 | cc. |

After all the salts were dissolved the two solutions were mixed, 2 drops of 10 per cent solution of $FeCl_3$ and a pinch of $CaCO_3$ added, and the whole heated to boiling to effect complete precipitation of the phosphates. Seven and one-half grams of Bacto agar were then added and dissolved with the aid of the autoclave. The solution was thoroughly stirred while hot and then filtered, tubed, and again autoclaved.

Agar II (phosphates allowed to precipitate after the addition of agar).

Solutions *A* and *B* were prepared exactly as above, and then 3.25 gms. agar dissolved in each, the solutions filtered, and then united, tubed, and autoclaved.

Agar III. This was the regular modified Ashby soil extract agar described above.

In tubing these agars 10-cc. portions were placed in Jena test-tubes containing a pinch of $CaCO_3$. Three slants of each agar were inoculated on the same date with triple strokes from a 72-hour culture of the $F_4$ generation. The results are shown in the following summary:

TABLE XII

SUMMARY OF GROWTH ON AGARS

| Agar no. | Growth after | | | |
|---|---|---|---|---|
| | 24 hours | 72 hours | 6 days | 10 days |
| I | Faint growths in 2 tubes, doubtful in 3rd | Raised streaks; fair growths, as compared with No. II distinctly less | Slimy, rather thin streaks; only one tube shows spreading; growths distinctly less than in No. II | Growths somewhat wrinkled and showing some pigmentation; growths slightly less than in No. II |

| Agar no. | Growth after | | | |
|---|---|---|---|---|
| | 24 hours | 72 hours | 6 days | 10 days |
| II | Faint growths | Pronounced, raised, smooth, slimy streaks | Growths somewhat flat and spreading, becoming dry and slightly wrinkled, dry portions becoming brownish | Good growths now dry and wrinkled; pigment production quite marked |
| III | Slight growths | Abundant rather flat growths, apparently best in whole series | Abundant, spreading growths; streaks quite largely run together; considerable invasion and collection of slime at base of slant | Abundant, flat, wrinkled growths; some pigment production; growths slightly heavier than in No. II |

The following general conclusions may be drawn from the above experiments with different agars: Agar No. I produces the poorest growth and No. III the best; agar No. II produces a growth distinctly better than No. I and almost as good as that on No. III.

*Nutrient solutions.*—In these experiments only enough agar was added to make the solution slightly viscous. Only one-tenth the amount of agar was used, i. e., 1.5 gms. per liter. Now, if this amount of agar functions as a protective colloid the precipitated phosphate should pass through the filter quite largely. That being true, then the effect of filtering the medium reported above (pp. 31–32) would largely disappear. To see whether any such action could be detected a modified Ashby medium was prepared, using .15 per cent agar as a protective colloid, and filtered and unfiltered portions tested with and without mechanical agitation. A modified Kaserer's solution was tested in a similar manner.

The modified Ashby solution was prepared as described above for the use of agar as a protective colloid, except that 0.75 gm. instead of 7.5 gms. was added to each of the solutions corresponding to solutions *A* and *B*. The two solutions were mixed, well stirred up with $CaCO_3$, heated for 30 minutes in the autoclave, and then a portion of the preparation filtered.

Twenty-five-cc. portions of the filtered and unfiltered medium were then placed in 1000-cc. "Nonsol" Erlenmeyer flasks containing a pinch of $CaCO_3$, plugged, capped with beakers, and autoclaved. The flasks were inoculated according to method *B* described previously (p. 27), the suspension being prepared from a 6-day-old slant of the $F_{18}$ culture. The cultures were incubated for 10 days at 28–30° C. Part of the flasks were placed on the mechanical shaker and part were kept on the shelf near by. The plan and results of the experiment are shown in table XIII.

TABLE XIII

MODIFIED ASHBY'S MEDIUM WITH .15 PER CENT AGAR AS PROTECTIVE COLLOID

| | No. | Treatment | Nitrogen (mgs.) |
|---|---|---|---|
| | | Unfiltered | |
| Shelf | 103* | Check | 0.17 |
| | 104* | Check | 0.13 |
| | 105 | Inoc. | 1.57 |
| | 106 | Inoc. | 1.70 |
| Shaker | 107 | Inoc. | 1.16 |
| | 108 | Inoc. | 1.39 |
| | | Filtered | |
| Shelf | 79 | Check | 0.16 |
| | 80 | Check | 0.12 |
| | 81 | Inoc. | 2.45 |
| | 82 | Inoc. | 2.39 |
| Shaker | 83 | Inoc. | 1.94 |
| | 84 | Inoc. | 2.30 |

* 300-cc. flasks used.

A duplicate experiment was carried out simultaneously, with all conditions the same except that no agar was used in the nutrient medium. The results appear in table XIV.

The results show that when a small amount of agar is added to a medium in such a way that it may act as a protective colloid, this medium then is not affected injuriously by filtering. On the other hand, if the agar be omitted, the filtered medium is distinctly inferior to the unfiltered. Moreover, in the presence of the agar the shaker is apparently of no benefit to the growth of the microörganisms.

TABLE XIV

MODIFIED ASHBY'S SOLUTION WITHOUT AGAR

| | No. | Treatment | Nitrogen (mgs.) |
|---|---|---|---|
| | | Unfiltered | |
| Shelf | 109* | Check | .07 |
| | 110* | Check | .03 |
| | 111 | Inoc. | 1.18 |
| | 112 | Inoc. | 0.98 |
| Shaker | 113 | Inoc. | 1.23 |
| | 114 | Inoc. | 1.31 |
| | | Filtered | |
| Shelf | 85 | Check | 0.16 |
| | 86 | Check | 0.08 |
| | 87 | Inoc. | 1.24 |
| | 88 | Inoc. | 0.24 |
| Shaker | 89 | Inoc. | 0.69 |
| | 90 | Inoc. | 0.45 |

* 300-cc. flasks used.

In a similar experiment using Kaserer's medium 2 per cent mannite was used as the energy source instead of the 1 per cent dextrose employed by Kaserer. Furthermore, the potassium silicate which we had available was so strongly alkaline that the medium had to be partially neutralized after this material was added. The medium was prepared as follows: One gm. $Al_2(SO_4)_3$ and 0.25 gm. of $FeCl_3$ were dissolved in approximately 350 cc. of distilled water, the solution heated, and the Fe and Al precipitated with $Na_2HPO_4$ solution. The precipitate was thrown down by means of a centrifuge, decanted, washed once by the same process, and then suspended in 700 cc. water containing 15 cc. of 10 per cent potassium silicate solution. The suspension was then strongly alkaline, and the required amount of N/10 acid as determined by titration was added to adjust the reaction to $P_H$ 8, the volume then made to 1 liter, distributed in bottles, and shaken on a machine for 4 hours. Complete solution was not effected. There was then added to the 1-liter portion:

| | |
|---|---|
| Mannite ..... | 20 gms. |
| Calcium sulphate ..... | .1 gm. |
| Manganese sulphate ..... | .1 gm. |
| Magnesium sulphate ..... | .1 gm. |
| Sodium chloride ..... | .1 gm. |

This suspension was then heated, and approximately one-half of it filtered through ordinary filter paper.

Twenty-five-cc. portions of the filtered and of the unfiltered medium were distributed into 1000-cc. "Nonsol" Erlenmeyer flasks, each containing a pinch of $CaCO_3$. Inoculations were made as described above from the same suspension and on the same date. Incubation was also under the same conditions and date. The results are reported in table xv.

TABLE XV

MODIFIED KASERER'S SOLUTION

| | No. | Treatment | Nitrogen (mgs.) |
|---|---|---|---|
| Unfiltered | | | |
| Shelf | 115* | Check | 0.11 |
| | 116* | Check | 0.05 |
| | 117 | Inoc. | 0.94 |
| | 118 | Inoc. | 0.95 |
| Shaker | 119 | Inoc. | 2.33 |
| | 120 | Inoc. | 2.40 |
| Filtered | | | |
| Shelf | 91* | Check | 0.07 |
| | 92 | Check | 0.06 |
| | 93 | Inoc. | 1.15 |
| | 94 | Inoc. | 1.54 |
| Shaker | 95 | Inoc. | 2.62 |
| | 96 | Inoc. | 2.72 |

* 300-cc. Erlenmeyer flasks used.

From the above data it appears that the filtered solution is fully as good as the unfiltered, and that mechanical stirring of the cultures is beneficial to both media. This indicates that both the filtered and the unfiltered media are poorly buffered and that the mechanical action of the shaker assists in the maintenance of the proper H ion concentration by hastening the solution of the calcium carbonate.

An attempt was made to conduct an experiment similar to the above with filtered and unfiltered solutions containing Remy and Rösing's colloidal ferric oxide, but the solution precipitated completely on heating, hence the results of the experiment were without significance.

## General Conclusions

The experimental work reported in this paper suggests that some of the markedly beneficial results observed in cultural solutions by different workers are associated with phosphorus nutrition of the organism and with maintenance of proper reaction of the medium. The experiments above on removal and restoration of the precipitate and on the use of glycerolphosphate, and those with protective colloids are suggestive, but do not yield the final proof of the mechanism of increased growth. The beneficial effect of the agar might be explained from the viewpoint of Kaserer, i. e., by the presence of certain nutrients in the agar, but this explanation seems less plausible than that of its action as a protective colloid.

Many experimental difficulties stand in the way of proper development of this interesting field of inquiry. Especially is this true in dealing with colloids. It is often difficult to duplicate the work of another investigator in the field of colloid chemistry, and this point is well illustrated by the contradictory results reported above with colloidal hydrated ferric oxide, in which case we were unable to duplicate even our own results. Moreover, the method of measuring growth at the end of a short incubation period, as has been done in the work reported in this paper, is wholly inadequate to permit a rigid examination of the results of different conditions. The method used by Bonazzi with the nitrite-producing bacteria, of repeatedly renewing the energy supply and measuring the products of growth, is far superior. If some such method could be used with *Azotobacter* a more reliable picture of the growth processes could be obtained.

The discussion of the arguments for and against the above theories might be extended greatly, yet this hardly seems to us worth while just at present, especially in view of the paucity of rigid experimental data. The working hypothesis suggested above may be of some help in the development of experimental work, and if subsequent experiments show it to be unsound it should be discarded. At present, however, it seems that there is fully as much in support of it as of the other

theories, and it has the advantage of being less roundabout; that is, the need of phosphates and the avoidance of an acid reaction are requirements of the culture medium, known beyond any doubt. It seems that the various ramifications of these *known* factors must be studied in detail before speculations in regard to "auximones" and "rare nutrients" be entered into widely.

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## Explanation of Plate

### PLATE 1

Photomicrographs of Azotobacter chroococcum, × 1170. Culture No. 5 from liquid medium, others from agar slants.

Fig. 1. A 12-hour-old culture stained with dilute aqueous methylene blue.

Fig. 2. Same, stained with carbol gentian violet.

Fig. 3. A 60-hour-old culture, methylene blue.

Fig. 4. Same, carbol gentian violet.

Fig. 5. A 5-day-old culture, methylene blue.

Fig. 6. A 15-day-old culture, carbol gentian violet.